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Biochimica et Biophysica Acta 1310 (1996) 60–66

BBA Biochimica et Biophysica Acta

Mastoparan elicits prostaglandin E_2 generation and inhibits inositol phosphate accumulation via different mechanisms in rabbit astrocytes

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Received 20 July 1995; accepted 9 August 1995

Abstract

The effects of mastoparan on phosphoinositide hydrolysis and prostaglandin E_2 (PGE_2) generation were investigated in astrocytes cultured from rabbit brain. Mastoparan inhibited the accumulations of [^{3}H]inositol phosphates induced by bradykinin (1 μM) in a time- and concentration-dependent manner. Mastoparan (3–30 μM) also released PGE_2 in a time- and concentration-dependent manner. Mastoparan-induced release of PGE_2 was inhibited by indomethacin, a cyclooxygenase inhibitor, by dexamethasone, a steroidal anti-inflammatory drug, and by pertussis toxin, an inactivator of some G proteins, such as Gi and Go. Mastoparan also caused [^{3}H]arachidonic acid liberation, which was inhibited by dexamethasone or pertussis toxin. In contrast, indomethacin, dexamethasone and pertussis toxin failed to attenuate mastoparan-induced inhibition of [^{3}H]inositol phosphate accumulation induced by bradykinin. Thus, mastoparan-induced inhibition of phosphoinositide hydrolysis does not involve pertussis toxin-sensitive G protein nor arachidonic acid metabolites. In addition to the inhibition of phospholipase C, mastoparan activates phospholipase A_2 through pertussis toxin-sensitive G protein.

Keywords: Astrocyte; Mastoparan; Phospholipase C; Phospholipase A_2; G protein; Prostaglandin E_2

1. Introduction

Mastoparan, a venom toxin composed of 14 amino acids, has several pharmacological actions, including histamine release from mast cells [1]. Since pertussis toxin eliminates mastoparan-induced histamine release [2], a GTP binding regulatory protein (G protein) may be involved in the actions of mastoparan. Higashijima et al. [3] demonstrate that mastoparan activates GTPase and GTPγS binding of purified G proteins, such as Go and Gi, in phospholipid vesicles. On the other hand, recent lines of evidence suggest that phosphoinositide hydrolysis mediated through pertussis toxin-sensitive G protein comes from an activation of phospholipase C-β2 by $\beta\gamma$ subunits dissociated from Gi [4,5]. Although many investigations have revealed that mastoparan activates phosphoinositide hydrolysis in several types of cells [6–8], we have previously demonstrated that mastoparan inhibits phosphoinositide hydrolysis in 1321N1 human astrocytoma cells [9]. Mastoparan also inhibits the increase in intracellular Ca^{2+} concentrations induced by carbachol or histamine [10], supporting the inhibitory effect of mastoparan on phosphoinositide hydrolysis. Similar results have been shown in permeabilized SH-SY5Y cells [11] and HL-60 cells [12]. However, it remains unknown why mastoparan action on phosphoinositide hydrolysis shows cell type discrepancy.

Astrocytes, which are assumed to exist 5–10 times more than neurons in brain, belong to a family of glial cells [13]. It has been reported that astrocytes have several receptors of neurotransmitters and autacoids [14–18], including arachidonic acid metabolites, such as thromboxane A_2 receptors [19]. 1321N1 human astrocytoma cells have also been shown to express thromboxane A_2 receptors [20] in addition to other Ca^{2+} mobilizing receptors, such as

Abbreviations: BSA, bovine serum albumin; DMEM, Dulbecco's modified eagle's medium; EMEM, Eagle's minimum essential medium; G-protein, guanine nucleotide regulatory protein; GTPγS, guanosine 5'-3-(thio)triphosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; MAPK, mitogen-activated protein kinase; PGE_2, prostaglandin E_2; TCA, trichloroacetic acid.

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SSDI 0167-4889(95)00145-X

muscarinic cholinergic [21], H_1-histamine [22], bradykinin [23] and endothelin [24], of which stimulations result in activation of phosphoinositide hydrolysis. On the other hand, astrocytes themselves release prostaglandins (PGs) in response to several stimuli, such as ATP [25].

In the present study, we cultured astrocytes from rabbit brain and examined the effect of mastoparan on phosphoinositide hydrolysis, to clarify the cell type discrepancy of mastoparan action on phosphoinositide hydrolysis. We also examined the effect of mastoparan on prostaglandin E_2 (PGE_2) generation in rabbit astrocytes. The results obtained suggest that mastoparan inhibits phosphoinositide hydrolysis in a pertussis toxin-insensitive manner in rabbit astrocytes, supporting the previous results in 1321N1 human astrocytoma cells. Furthermore, mastoparan activates phospholipase A_2 through pertussis toxin-sensitive G protein.

2. Materials and methods

2.1. Materials

Mastoparan was purchased from Peptide Chemistry (Osaka, Japan). Bradykinin was from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and Eagle's minimum essential medium (EMEM) were obtained from Nissui Pharmaceuticals (Tokyo, Japan). Fetal calf serum (FCS) was from Cell Culture Laboratory (Cleveland, OH, USA). Pertussis toxin was from Funakoshi (Tokyo, Japan). Indomethacin was from Wako Pure Chemicals (Osaka, Japan). Dexamethasone was from Banyu Pharm. (Tokyo, Japan). Prostaglandin E_2 (PGE_2) and anti-PGE_2 antibody were generous gifts from Ono Pharmaceuticals (Osaka, Japan). [^{3}H]Myo-inositol was purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). [^{3}H]PGE_2 was from NEN/DuPont (Boston, MA, USA). [^{3}H]Arachidonic acid was from Moravek Biochemicals (Brea, CA, USA).

2.2. Cell culture

Rabbit astrocytes were cultured as described previously [19]. Briefly, the cerebral cortex of rabbit (2.5–3.0 kg) was cut into small pieces with scissors, and was treated with trypsin (0.02%) for 30 min. Then, the cells filtered by a nylon mesh were collected by centrifugation at $200 \times g$ for 1 min and washed several times with DMEM buffered with 20 mM Hepes (pH 7.4). Then cells were suspended in DMEM supplemented with 10% FCS in a concentration of about 10^7/ml, and cultured in an incubator containing 5% CO_2 at 37°C. Cells were shaken for 24 h to remove oligodendrocytes 10–14 days after starting the culture. The medium was changed every 3–4 days. After 2–3 weeks, over 90% of the cells were astrocytes determined by anti-glial fibrillary acidic protein antibody.

2.3. Assay of [^{3}H]inositol phosphates

Phosphoinositide hydrolysis was monitored by determination of [^{3}H]inositol phosphates, described previously [9,20,26]. In brief, rabbit astrocytes were seeded into 12-well plates at the density of 10^5 cells per well. Two days after seeding cells, the medium was changed to DMEM containing 2 μCi/ml of [^{3}H]inositol for 18 h. The reaction was started by adding drugs in Eagle's minimum essential medium buffered by 20 mM Hepes to pH 7.35 (EMEM-Hepes) in the presence of 10 mM LiCl. The reaction was terminated by addition of 5% trichloroacetic acid (TCA) after aspiration of the medium. The TCA extract was washed three times with ether, and applied to an anion exchange column (AG 1X-8, formate form). Total [^{3}H]inositol phosphates were eluted by 1 M ammonium formate in 0.1 M formic acid, and counted by liquid scintillation counting.

2.4. Assay of prostaglandin E_2

PGE_2 was analyzed by radioimmunoassay, described previously [27]. In brief, the cells were seeded into 12-well plates at the density of 10^5 cells per well. Two days after seeding cells, the cells were washed twice with EMEM-Hepes (pH 7.35), then the cells were preincubated for 10 min at 37°C. After the cells were incubated with drugs for an additional 10 min, the medium was transferred into a tube. The medium containing PGE_2 was stored at −20°C until radioimmunoassay. The medium was acidified to pH 4.0 by adding 1 N HCl, and PGE_2 was extracted twice by ethyl acetate. After drying ethyl acetate under reduced pressure, the sample was dissolved in 10 mM Tris-HCl (pH 7.6). The sample was incubated overnight at 4°C with [^{3}H]PGE_2 (10000 dpm) and anti-PGE_2 antibody (450 times dilution) in a final volume of 300 μl containing 0.5% bovine serum albumin (BSA) in 100 mM Tris-HCl (pH 7.6). The free [^{3}H]PGE_2 was sedimented by centrifugation after adding 0.5 ml of the buffer containing 0.5% charcoal, 0.075% dextran and 0.5% BSA in 100 mM Tris-HCl (pH 7.6). The [^{3}H]PGE_2 bound to antibody was counted by liquid scintillation counting.

2.5. Arachidonic acid liberation

The cells were seeded into 6-well plate at the density of 2×10^5 cells per well. Three days after seeding cells, the cells were labeled with 3 μCi/ml of [^{3}H]arachidonic acid for 18 h. Then, the cells were washed twice with EMEM-Hepes (pH 7.35), and were preincubated for 10 min. After incubation with drugs for 10 min, the incubation medium was transferred to the tube containing an equi-volume of ice-cold chloroform/methanol/1 N HCl (10/20/1). [^{3}H]Arachidonic acid in chloroform was separated by thin-layer chromatography, using a silica gel plate (LK5D, Whatman Inc.) and a developer of benzene/dioxane/acetic

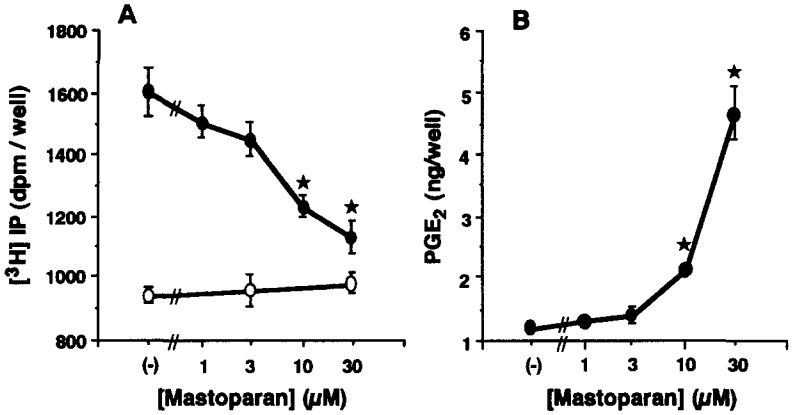

Fig. 1. Effect of mastoparan on phosphoinositide hydrolysis and prostaglandin E_2 generation in rabbit cultured astrocytes. (A) The cells were labeled with 2 μCi/ml of [^{3}H]inositol for 18 h. After washing, the cells were incubated with mastoparan in the presence (●) or absence (○) of 1 μM of bradykinin for 10 min. Analysis of [^{3}H]inositol phosphates (IP) was described in Section 2 in detail. Each point represents the mean ± S.E. from three determinations. * Significant difference from bradykinin alone ($P < 0.05$). (B) The cells were incubated with mastoparan at the indicated concentration for 10 min. Determination of prostaglandin E_2 (PGE_2) was described in Section 2 in detail. Each point represents the mean ± S.E. from three determinations. * Significant difference from without mastoparan ($P < 0.05$).

acid = 60/30/3. The portion corresponding to authentic arachidonic acid was scraped and counted by liquid scintillation counting.

2.6. Drug treatment

Dexamethasone or pertussis toxin was added to the culture medium 18 h before the experiment. When the cells were labeled with [^{3}H]inositol or [^{3}H]arachidonic acid, the drug was added simultaneously. The cells treated with dexamethasone or pertussis toxin were washed twice with EMEM-Hepes (pH 7.35) before starting the experiments. Indomethacin was added 20 min before the addition of other drugs.

2.7. Data analysis

The results obtained were expressed as mean ± S.E., and a significant difference ($P < 0.05$) was analyzed with Student's *t*-test.

3. Results

3.1. Effect of mastoparan on phosphoinositide hydrolysis and prostaglandin E_2 release in rabbit astrocytes

Stimulation of bradykinin receptor elicited phosphoinositide hydrolysis in rabbit astrocytes (Fig. 1A), similar

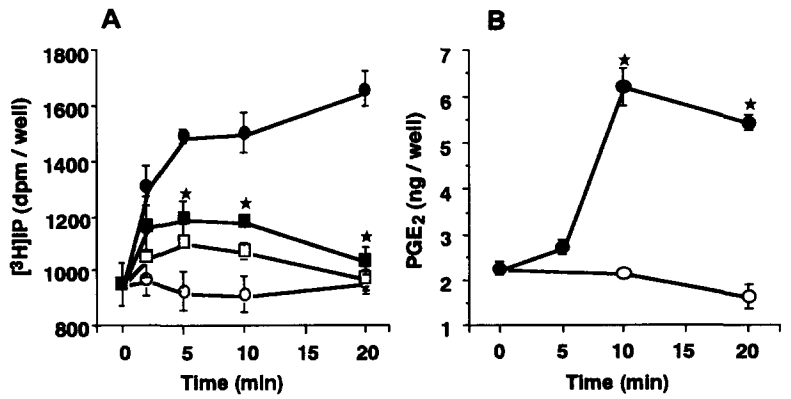

Fig. 2. Time course of mastoparan-induced inhibition of phosphoinositide hydrolysis and prostaglandin E_2 generation. (A) The cells were labeled with 2 μCi/ml of [^{3}H]inositol for 18 h. After washing, the cells were incubated with mastoparan in the presence (■) or absence of (□) of 1 μM bradykinin, or withour mastoparan in the presence (●) or absence (○) of 1 μM bradykinin. Each point represents the mean ± S.E. from three experiments. * Significant difference between bradykinin alone and bradykinin plus mastoparan ($P < 0.05$). (B) Cultured rabbit astrocytes were incubated with (●) or without (○) 30 μM mastoparan for indicated time. Each point represents the mean ± S.E. from three determinations. The prostaglandin E_2 (PGE_2) generation was maximum after 10 min incubation with mastoparan. * Significant difference from without mastoparan at each time ($P < 0.05$).

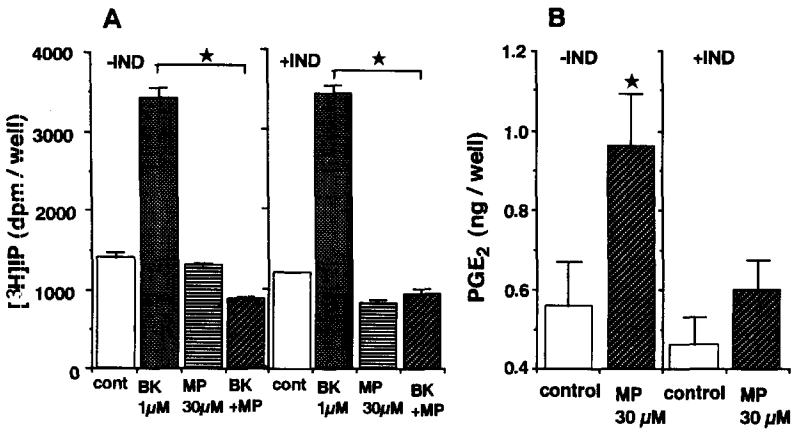

Fig. 3. Effect of indomethacin on mastoparan-induced inhibition of phosphoinositide hydrolysis and prostaglandin E_2 generation. (A) Inhibition of phosphoinositide hydrolysis by mastoparan (MP). Left: in the absence of indomethacin (IND). MP at the concentration of 30 μM significantly inhibited bradykinin (BK)-induced phosphoinositide hydrolysis (* $P < 0.05$). Right: MP also significantly inhibited BK-induced phosphoinositide hydrolysis in the presence of 5 μM IND (* $P < 0.05$). (B) Prostaglandin E_2 (PGE_2) generation induced by 30 μM MP. Left: in the absence of IND. Right: in the presence of 5 μM IND. * Significant difference ($P < 0.05$) from control. Note the IND attenuated MP-induced PGE_2 generation. Each column represents the mean with S.E. from three determinations.

to in human astrocytoma cells [23]. Mastoparan in a concentration range of 3–30 μM did not activate or only slightly activates phosphoinositide hydrolysis, but it inhibited bradykinin-induced phosphoinositide hydrolysis in a concentration-dependent manner (Fig. 1A). Time course analysis revealed that mastoparan at a concentration of 30 μM inhibited bradykinin-induced phosphoinositide hydrolysis within 5 min (Fig. 2A). The inhibition was clearly observed between 5–20 min. On the other hand, mastoparan released PGE_2 from astrocytes in a concentration-dependent manner (Fig. 1B). Mastoparan-induced PGE_2 release appeared with the maximum of 10 min (Fig. 2B). Mastoparan also inhibited ATP-induced phosphoinositide hydrolysis (Table 1), suggesting that the inhibitory action of mastoparan might not be due to a blockade of bradykinin receptor.

3.2. *Effect of indomethacin on mastoparan-induced inhibition of phosphoinositide hydrolysis and prostaglandin E_2 release*

Since mastoparan stimulates PGE_2 release in addition to inhibition of phosphoinositide hydrolysis in rabbit astrocytes, the role of arachidonic metabolites on phosphoinosi-

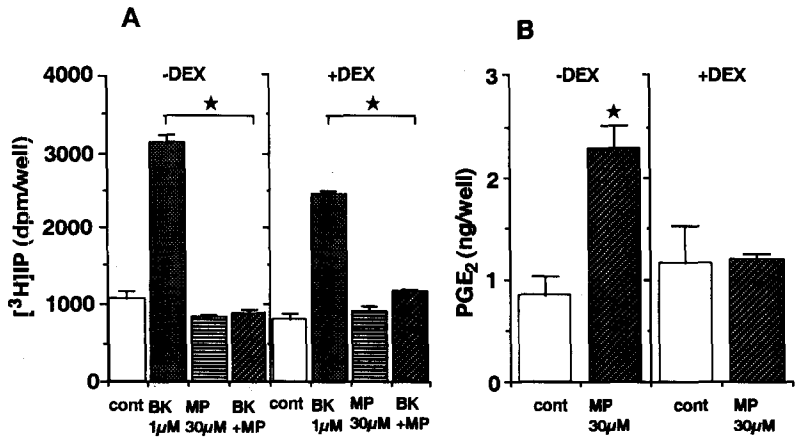

Fig. 4. Effect of dexamethasone on mastoparan-induced inhibition of phosphoinositide hydrolysis and prostaglandin E_2 generation (A) Inhibition of phosphoinositide hydrolysis by mastoparan (MP). Left: MP at the concentration of 30 μM significantly inhibited 1 μM bradykinin (BK)-induced phosphoinositide hydrolysis in the cells without dexamethasone (DEX) treatment (* $P < 0.05$). Right: MP also inhibited phosphoinositide hydrolysis significantly after the cells were treated with DEX for 18 h (* $P < 0.05$). (B) Prostaglandin E_2 (PGE_2) generation induced by 30 μM MP. Left: MP (30 μM) significantly (* $P < 0.05$) stimulated PGE_2 generation in the cells without treatment of DEX. Right: Effect of MP (30 μM) after treatment of the cells with DEX (100 ng/ml) for 18 h. Note that DEX attenuated MP-induced PGE_2 generation. Each column represents the mean with S.E. from three determinations.

Table 1
Effect of mastoparan on ATP-induced phosphoinositide hydrolysis

Addition	[^{3}H]Inositol phosphates (dpm/well)
control	833.1 ± 47.1
Mastoparan (30 μM)	1184.9 ± 49.0
ATP (100 μM)	4949.9 ± 49.4 [a]
Mastopran (30 μM) +ATP (100 μM)	2754.5 ± 52.3 [a,b]

Cells were labeled with [^{3}H]inositol (2 μCi/ml) for 18 h, and were incubated with mastoparan (30 μM) and/or ATP (100 μM) for 10 min. Values represent mean ± S.E. from three determinations. [a] Significant difference from control ($P < 0.05$); [b] significant difference from ATP alone ($P < 0.05$).

tide hydrolysis was investigated (Fig. 3). Indomethacin (5 μM) potently inhibited mastoparan-induced PGE_2 release (Fig. 3B), while it did not modify the inhibitory effect of mastoparan on bradykinin-induced phosphoinositide hydrolysis (Fig. 3A).

3.3. Effect of dexamethasone on mastoparan-induced inhibition of phosphoinositide hydrolysis and prostaglandin E_2 release

The treatment of the astrocytes with dexamethasone, a synthetic steroid hormone used as an anti-inflammatory drug, for 18 h, did not modify the mastoparan-induced inhibition of phosphoinositide hydrolysis elicited by bradykinin, although it slightly inhibited bradykinin-induced phosphoinositide hydrolysis (Fig. 4A). However, dexamethasone potently inhibited mastoparan-induced PGE_2 release (Fig. 4B).

3.4. Effect of pertussis toxin on mastoparan-induced inhibition of phosphoinositide hydrolysis and prostaglandin E_2 release

The treatment of the cells with pertussis toxin for 18 h resulted in a slight potentiation of bradykinin-induced phosphoinositide hydrolysis (Fig. 5A). The results indicate that bradykinin activates phospholipase C in a pertussis toxin-insensitive manner. Furthermore, pertussis toxin did not change mastoparan-induced inhibition of phosphoinositide hydrolysis elicited by bradykinin (Fig. 5A). Pertussis toxin was potently effective in attenuating PGE_2 release in response to mastoparan (Fig. 5B). Pertussis toxin at the concentration of 3 ng/ml caused about 60% reduction of mastoparan-induced PGE_2 release, indicating the involvement of a pertussis toxin-sensitive G protein in PGE_2 release by mastoparan.

3.5. Effects of dexamethasone and pertussis toxin on mastoparan-induced arachidonic acid liberation

Mastopran at a concentration of 30 μM liberated arachidonic acid significantly (Fig. 6). Pretreatment of cells with dexamethasone (100 ng/ml) for 18 h resulted in the attenuation of mastopran-induced arachidonic acid liberation. The inhibitory potency of dexamethasone in arachidonic acid liberation was weaker than that in PGE_2 release (Fig. 4). Furthermore, the treatment of cells with

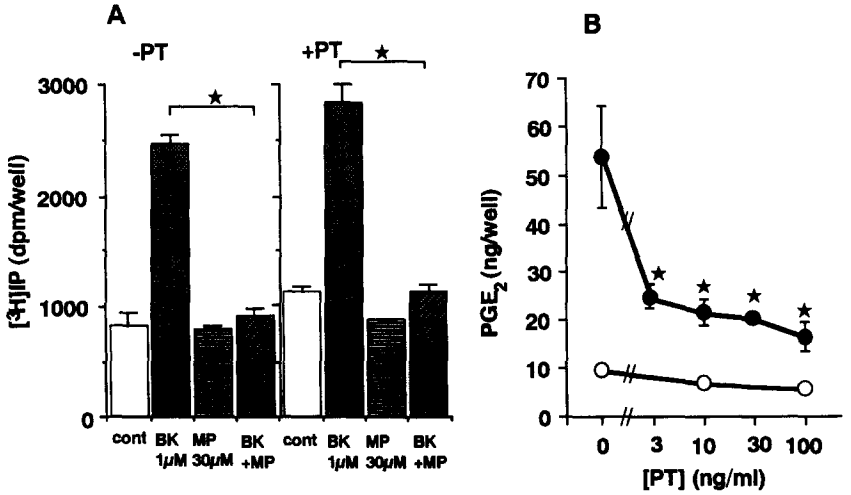

Fig. 5. Effect of pertussis toxin on mastoparan-induced inhibition of phosphoinositide hydrolysis and prostaglandin E_2 generation. (A) Inhibition of phosphoinositide hydrolysis by mastoparan (MP). Left: MP at the concentration of 30 μM significantly inhibited 1 μM bradykinin (BK)-induced phosphoinositide hydrolysis in the cells without pertussis toxin (PT) treatment (* $P < 0.05$). Right: MP also significantly inhibited phosphoinositide hydrolysis after the cells were treated with PT (100 ng/ml) for 18 h (* $P < 0.05$). (B) Effect of PT on prostaglandin E_2 (PGE_2) generation induced by 30 μM MP. The cells were treated with PT at the concentrations of 3–100 ng/ml for 18 h. * Significant difference from MP alone without PT treatment ($P < 0.05$). Note that PT attenuated MP-induced PGE_2 generation. Each column represents the mean with S.E. from three determinations.

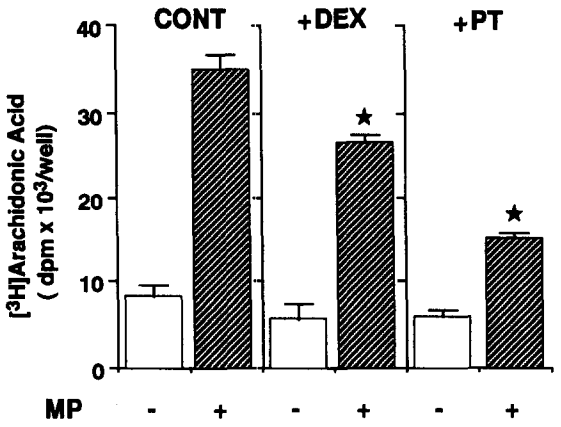

Fig. 6. Effects of dexamethasone and pertussis toxin on mastopran-induced arachidonic acid liberation. Left: Liberation of [^{3}H]arachidonic acid by 30 μM mastoparan (MP). Middle: liberation of [^{3}H]arachidonic acid by 30 μM mastoparan (MP) from the cells treated with dexamethasone (DEX, 100 ng/ml). DEX significantly inhibited MP-induced liberation of [^{3}H]arachidonic acid (* $P < 0.05$). Right: liberation of [^{3}H]arachidonic acid by 30 μM mastoparan (MP) from the cells treated with pertussis toxin (PT, 100 ng/ml). PT significantly inhibited MP-induced liberation of [^{3}H]arachidonic acid (* $P < 0.05$). Each column represents the mean with S.E. from three determinations.

pertussis toxin (100 ng/ml) for 18 h potently attenuated mastoparan-induced arachidonic acid liberation.

4. Discussion

The present study demonstrates that mastoparan inhibits agonist-induced phosphoinositide hydrolysis in rabbit astrocytes, consistent with the previous results in 1321N1 human astrocytoma cells [9,10]. In addition, mastoparan stimulates PGE_2 release in rabbit astrocytes. Cyclooxygenase products are not involved in the mastoparan-induced inhibition of phosphoinositide hydrolysis, because indomethacin does not affect the inhibition. Mastoparan-induced PGE_2 release and inhibition of phosphoinositide hydrolysis have different mechanisms that can be discriminated by pertussis toxin and/or dexamethasone.

Although mastoparan stimulates phosphoinositide hydrolysis in mast cells [6], PC-12 cells [8], rat hepatocytes [28] and rat parotid cells [7], it clearly inhibits phosphoinositide hydrolysis in rabbit astrocytes as well as in human astrocytoma cells [9]. Recent lines of evidence suggested that there are three pathways of phospholipase C activation. The first is tyrosine kinase-mediated activation of phospholipase C-γ isozyme [29]. This pathway does not involve G protein, and is activated by growth factors. The second is phospholipase C-β activation by $\beta\gamma$ subunits of Gi, which is a pertussis toxin substrate [4,5]. The third is phospholipase C-β_1 activation by the α-subunit of $G_{q/11}$, which is not a substrate for pertussis toxin [30–32]. Since mastoparan has been shown to activate pertussis toxin-sensitive G protein selectively such as Gi and Go [3], and Gi-mediated phospholipase C activation is lacking in rabbit astrocytes and human astrocytoma cells, then mastoparan does not activate phospholipase C in these cells. The exact mechanism of the inhibition of phosphoinositide hydrolysis by mastoparan in these cells remains unknown, so far. But there are a few possible mechanisms in the inhibition. The first is that the composition of phospholipids in membranes of astrocytes and/or astrocytoma cells may be different from mast cells, and the pharmacological activity of mastoparan may be dependent on phospholipid composition, suggested by Raynor et al. [33]. The second is that mastoparan inhibits directly a Ca^{2+} requiring phospholipase C by interfering with Ca^{2+} utilization. In fact, it has been reported that mastoparan inhibits Ca^{2+}/calmodulin [34]. However, mastoparan inhibits GTPγS-induced activation of phospholipase C more potently than Ca^{2+} (1 mM)-induced activation in membrane preparations of human astrocytoma cells [9] and HL-60 cells [12]. The third is that mastoparan activates a G protein that inhibits phospholipase C. To elucidate the exact mechanism of mastoparan-induced inhibition of phosphoinositide hydrolysis, further studies are necessary.

Mastoparan activates phospholipase A_2, resulting in a liberation of arachidonic acid and a generation of PGE_2 in rabbit cultured astrocytes, as it does in fibroblasts [35,36]. Because dexamethasone inhibits PGE_2 release more potently than arachidonic acid liberation, dexamethasone may inhibit cyclooxygenase in addition to phospholipase A_2. In fact, it has been shown that dexamethasone inhibits the gene expression of prostaglandin endoperoxide-2 (inducible cyclooxygenase) in mesangial cells [37]. Since the arachidonic acid liberation and the PGE_2 release are inhibited by pertussis toxin, the site of action of mastoparan may be a G protein, which in turn secondarily activates phospholipase A_2. G protein-mediated activation of phospholipase A_2 in response to receptor stimulation has been shown in other cells, where the activation was also inhibited by pertussis toxin [38]. Although mastoparan is not a receptor agonist, it directly activates G proteins, like an activated receptor, as suggested by Higashijima et al. [3]. Our present finding, that mastoparan-induced arachidonic acid liberation and PGE_2 release are potently inhibited by pertussis toxin, supports the idea that mastoparan directly activates G protein. Therefore, mastoparan is a useful tool for analyzing the interaction of an activated receptor and G protein in a model system. On the other hand, recent lines of evidence have suggested that mitogen-activated protein kinase (MAPK) is involved in the process of phospholipase A_2 activation [39], and MAPK is activated through Gi and $p21^{ras}$ pathway [40]. Because pertussis toxin inhibits mastoparan-induced arachidonic acid liberation and PGE_2 release (Fig. 5), one of the mechanisms of mastoparan action might be through Gi-MAPK and phospholipase A_2. Since mastoparan still activates arachidonic acid liberation to a small extent even in the cells treated with high concentrations of pertussis toxin, further studies

are necessary to clarify the signal transduction of mastoparan-induced PGE_2 release in detail. In conclusion, mastoparan inhibits phosphoinositide hydrolysis in rabbit astrocytes with different mechanisms from activation of a pertussis toxin-sensitive G protein, and mastoparan also activates arachidonic acid liberation and PGE_2 release through a pertussis toxin-sensitive G protein.

Acknowledgements

We are grateful to Ms. Mizuyo Kutsuwa, Tohoku University, for her technical assistance and discussion, and to Ono Pharmaceuticals for the generous gift of prostaglandin E_2 and its antibody. This work is partly supported by a Grant-in-Aid for Scientific Research (04304048, 05271102 and 05671805 to N.N.) from the Ministry of Education, Science and Culture of Japan.

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